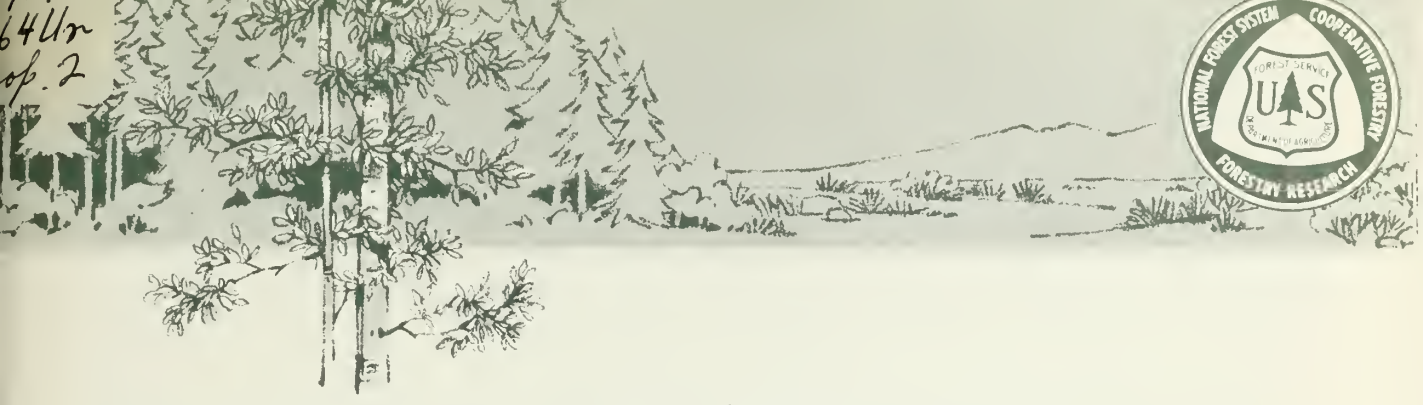

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CALLUS PROLIFERATION AND DIFFERENTIATION OF *RIBES* SPP. *IN VITRO*

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ABSTRACT

Media satisfactory for the induction of callus proliferation from primary explants of Ribes nigrum, R. petiolare, *and* R. viscosissimum *contained (g/l); calcium nitrate 0.5, magnesium sulfate 0.14, potassium phosphate (monobasic) 0.14, ammonium sulfate 0.025, ferric sulfate 0.014, manganese sulfate 0.0035, sucrose 30.0, Noble agar 10.0, kinetin 0.001, plus IAA 0.1, NAA 0.01, or 2,4-D 0.00015 (pH 6.0). This medium maintained callus proliferation for periods up to 6 months with one subculture. Additions of myo-inositol 0.0002, ascorbic acid 0.0002, Biotin 0.00001, thiamine 0.0001, and pyrodoxine 0.0001 extended explant longevity but were not required. Primary explants and first subcultures of* R. nigrum *and* R. petiolare, *on the complete medium plus IAA (g/l), 0.0001, 0.001 and 0.01, formed roots, roots and shoots, and shoots, respectively. Only roots were formed in the absence of kinetin.*

KEYWORDS: *Ribes*, tissue culture, callus proliferation, differentiation, root induction, shoot induction

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Studies of the host-parasite interactions of the mononucleate stage of *Cronartium ribicola* J.C. Fisch. ex Rabenh. in tissue cultures of *Pinus monticola* Dougl. (Harvey and Grasham 1969; Harvey and Woo 1971; Robb and others 1974a, 1974b) have provided basic knowledge concerning the histopathological and nutritional interactions of these two organisms. Tissue cultures of the *Ribes* host would further studies of the binucleate stage. Comparison of the two should provide a better understanding of the similarities or differences between the nuclear stages of this rust, and perhaps a clearer understanding of each. This paper describes procedures and media for inducing callus proliferation and differentiation of *Ribes nigrum* L., *R. petiolare* Dougl., and *R. viscosissimum* Pursh. Insofar as possible, the basic approach paralleled that previously described for the pine host of this important parasite (Harvey 1967; Grasham and Harvey 1970).

MATERIALS AND METHODS

Primary explants were prepared from either 2-cm-long stem sections or 0.5-cm-long petiole sections taken from current year's shoots of 3- to 5-year-old, greenhouse-grown, potted plants or wildings of the three species under study. Sections of either tissue from all species were rinsed in rapidly running water for 2 h and sterilized for 10 min (with constant agitation) in 0.525 percent sodium hypochlorite. All tissues were subsequently rinsed in three changes of sterile, distilled water. Petiole sections, after excising 1 mm pieces from both ends to remove injured tissues, were planted by embedding the acropetal end 0.25 cm into an experimental medium. Stem sections were taken from the internodes between the fifth and eighth leaf below the actively growing meristem. In this area the cortex layer was well developed but lenticular tissues were poorly developed and were not visible on the external surface. Surface-sterilized sections were further prepared by aseptically removing the epidermis, cutting into 0.5-cm lengths, and planting as above, or by subsequently removing the cortex layer from the pith and dividing the resultant sheets of stem cortex into 1-cm squares. These were placed, internal surface down, on an experimental medium.

Media were developed by adding an auxin and kinetin (6-furfurylaminopurine) in specified ratios to vitamins, amino acids, casein hydrolysate, peptone and yeast extract, singly or in combinations, at various concentrations, to the basal medium previously developed for *P. monticola* (Harvey 1967; Harvey and Grasham 1969; Grasham and Harvey 1970). This basal medium contained (g/l); calcium nitrate 0.5, magnesium sulfate 0.14, potassium phosphate (monobasic) 0.14, ammonium sulfate 0.025, ferric sulfate 0.014, manganese sulfate 0.0035, sucrose 30.0, and Noble agar 10.0 (table 1). All organic additives were filter sterilized prior to incorporation into the autoclaved (15 lb for 20 min) basal medium (50° C).

Callus cultures were incubated under a 24-h cyclic regimen: 16 h at 21° C, 400 footcandles, and 8 h at 5° C, no light.

All media were prepared with double-distilled water (pH 6.5); the second distillation was in an all-pyrex-and-Teflon-glass still.

RESULTS

Excellent callus proliferation was achieved with all three types of primary explants from all three species on the basal medium described amended with (g/l) indoleacetic acid (IAA) 0.1, napthaleneacetic acid (NAA) 0.01, or 2,4-dichlorophenoxyacetic acid (2,4-D) 0.00015, in combination with kinetin 0.001. IAA, NAA, and 2,4-D at concentrations greater than 0.1, 0.01, and 0.0005, respectively, were toxic. IAA and NAA were inferior to 2,4-D. See example of *R. nigrum* in table 1. The former caused etiolation and necrosis of all three species after 45 days. In addition, subculturing of callus tissues grown on these two auxins was difficult.

Table 1.--*Relative efficiency of various auxin/kinetin combinations, added to the basal medium, in inducing callus growth, root and shoot formation 60 days after explanting 1-cm squares of stem cortex from* R. nigrum

Acid	Auxin	Kinetin	Relative abundance[1]			
			Callus growth	Roots	Shoots	Callus necrosis
	g/l					
1AA	0.0001	0.001	+	++	0	+
1AA	0.001	0.001	++	++	++	+
1AA	0.01	0.001	++	0	0	+
1AA	0.1	0.001	+++	0	0	+
NAA	0.01	0.001	+++	0	0	+
2,4-D	0.00015	0.001	+++++	0	0	0

[1] Rated from 0 to +++++ where the latter was optimum, intermediate levels based on visual comparisons.

Excellent proliferation was produced by primary explants derived from the 1-cm^2 sections of stem cortex. General vigor of the three types of explants of all three species was enhanced by the addition of (g/l) myo-inositol 0.0002, ascorbic acid 0.0002, biotin 0.00001, thiamine 0.0001, and pyrodoxine 0.0001, particularly in the presence of vitamin-free casein hydrolysate 0.01, bacto-peptone 0.50, or yeast extract 0.50.

Callus growth was firm but friable; and, although chlorophyll production was evident in the first 30 days, the proliferating tissues generally became etiolated. Only tissues derived from excised stem cortex continued to proliferate for periods in excess of 90 days without subculture. This tissue acquired a zonate pattern of growth with recurring proliferation approximately every 45 days (fig. 1a). Histological examination revealed recurring meristematic layers (fig. 1d). All callus tissues consisted of large, thin-walled cells organized into loosely cemented tissue masses (fig. 1f).

Subcultures of 15-mm^3 cubes of healthy callus (without primary tissue) from any of the three explant types from *R. nigrum* and *R. petiolare* could be maintained, by continuous subculture of vigorous material, for periods up to 6 months (five to eight generations). Frequently, extensive necrosis prevented additional transfers after 90 days. Explants from *R. viscosissimum*, particularly excised stem cortex, proliferated rapidly for the first 60 days, but degenerated if not subcultured at about 21-day intervals. Petiole-derived callus from all three species was slow growing and became necrotic after 90 days without subculture.

Differentiation of roots and shoots in primary explants or the first subculture of *R. nigrum* and *R. petiolare* was easily obtained by varying IAA/kinetin ratios (table 1). However, after the first transfer, callus tissues did not differentiate. NAA or 2,4-D maintained cultures did not differentiate. Figures 1b, e, and c are typical of the differentiation patterns obtained with primary explants or first subcultures propagated on the basal medium plus (g/l) kinetin 0.001 and IAA at 0.0001, 0.001, and 0.01, respectively. These examples were obtained from stem cortex primary explants. Pith tissues had not been removed. Photographs were taken 90 days after beginning the cultures. IAA at (g/l) 0.1 supported callus proliferation but inhibited both root and shoot formation. In the absence of kinetin, differentiation rarely occurred from proliferating callus tissues. In those cases where it did occur, only roots were formed.

Figure 1.--(a) Callus development from a stem cortex explant after 120 days, without subculture, 2,4-D (g/l) 0.00015. Note the zonate growth pattern. (b) Root formation in a stem segment primary explant after 60 days, 0.0001 IAA and 0.001 kinetin. (c) Shoot formation in a stem segment primary explant after 60 days, 0.01 IAA and 0.001 kinetin. (d) Cross section through callus shown in 1a, 200X. Note recurring meristematic layers. (e) Root and shoot formation in a stem segment primary explant after 60 days, 0.001 IAA and 0.001 kinetin. (f) Cross section of the large thin-walled callus cells produced from a stem cortex explant after 30 days, 400X. Cross sections prepared in paraffin and stained with Sass' triple stain.

a
b
KIMAX
c
d
e
KIMAX
f

DISCUSSION

Although the mineral salts and organic compounds essential to induce callus proliferation of the three *Ribes* species are relatively few, it is apparent that one or several of the compounds used in our test media are either limiting, toxic, or both; or the media are deficient in some other essential nutrient. However, careful preparation of subcultures, to remove all necrotic tissues formed in the previous transfer, was helpful for long-term propagation. Because tissues were grown in an environment that provided periodic light, the gradual etiolation as cultures aged would appear to be related to the onset of necrosis.

One could speculate that the zonate growth pattern exhibited by *Ribes* tissue cultures, most evident in callus derived from stem cortex, may be related to an endogenous circadian cycle. Such a cycle may also interfere with continuous growth in culture if the dormancy requirements of the species have not been compensated for in the artificial environment.

The ability of *Ribes* callus to differentiate roots and shoots was severely inhibited by continuous subculture. Callus propagated beyond the first subculture rarely differentiated and, if it did, roots and not shoots were formed. Similar loss of the capability to differentiate has also been reported for *Geranium* callus (Pillai and Hildebrandt 1969).

Auxin levels required for the propagation of *Ribes* sp., a woody perennial shrub, were slightly lower than those required for many forest tree species, including both woody dicots (Jacquiot 1964; Wolter 1964; Wolter and Skoog 1966; Winton 1968) and conifers (Harvey 1967; Brown and Lawrence 1968; Harvey and Grasham 1969). This may be indicative of differences in the endogenous auxin levels correlated directly or indirectly to physical stature, apical dominance, or both.

The differentiation pattern of *Ribes* callus was directly related to IAA/kinetin ratios in the medium. This pattern was characterized by root formation at low auxin/kinetin ratios (g/l) (0.0001/0.001), both root and shoot formation at intermediate ratios (0.001/0.001), and shoot formation at high ratios (0.01/0.001), followed by complete inhibition of differentiation at high auxin concentrations (0.1). IAA became toxic beyond 0.1 g/l. This general pattern of auxin/kinetin control of differentiation was reported for aspen callus (Winton 1968), but the auxin concentrations used were somewhat higher. For herbaceous dicots, the pattern was different and frequently reversed (Pillai and Hildebrandt 1969; Skoog and Miller 1957; Torrey and Sigemura 1957; Vasil and Hildebrandt 1966a, 1966b; Wilmar and Hellendorn 1968; Halperin 1969). Thus, it is apparent that a great many endogenous factors and environmental requirements control the expression of growth-regulator-induced differentiation in culture.

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